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The C-terminal tail of polycystin-1 regulates complement factor B expression by signal transducer and activator of transcription 1

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Abstract: Inhibition of the overactivated alternative complement pathway in autosomal dominant polycystic kidney disease (ADPKD) retards disease progression in animal models; however, it remains unknown how complement factor B (CFB) is upregulated in ADPKD. Here, we showed that the overexpression of CFB in cystic kidneys is associated with increased JAK2/STAT1 activity and enhanced expression of the polycystin-1 C-terminal tail (PC1-CTT). Overexpression or blockage of STAT1 increased or decreased CFB expression and CFB promoter activity. Moreover, overexpression of PC1-CTT induced JAK2/STAT1 activation and CFB upregulation in renal tubular epithelial cells. Furthermore, PC1-CTT overexpression increased human CFB promoter activity, whereas dominant negative STAT1 plasmids or mutation of putative STAT1 responsive elements decreased PC1-CTT-induced CFB promoter activity. The effect of CFB on macrophage differentiation was tested on a mouse macrophage cell line. Bioactive CFB dose dependently promoted macrophage M2 phenotype conversion. In addition, conditioned media from renal epithelial cells promoted macrophage M2 phenotype conversion which was blocked by STAT1 inhibition in a dose-dependent manner. Conditioned media from PC1-CTT-transfected renal epithelial cells further promoted macrophage M2 phenotype conversion, which was suppressed by fludarabine or a CFB antibody. In addition, we show that NF- B acts downstream of PC1-CTT and may partly mediate PC1-CTT-induced CFB expression. In conclusion, our study reveals possible mechanisms of CFB upregulation in ADPKD and a novel role of PC1-CTT in ADPKD-associated inflammation. Furthermore, our study suggests that targeting STAT1 may be a new strategy to prevent inflammation in the kidney of patients with ADPKD.

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The C-terminal tail of polycystin-1 regulates complement factor B expression by Signal Transducer and Activator of Transcription 1

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Running head: PC1 promotes CFB expression through STAT1

Keywords: kidney; Complement; macrophage; Polycystic Kidney Disease (PKD); polycystin-1 C-terminal tail (PC1-CTT); Janus kinase 2 (JAK2); signal transducer and activator of transcription 1 (STAT1)

Abstract

Inhibition of the over-activated alternative complement pathway in autosomal dominant polycystic kidney disease (ADPKD) retards disease progression in animal models, however it remains unknown how complement factor B (CFB) is up-regulated in ADPKD. Here we showed that the overexpression of complement factor B (CFB) in cystic kidneys associated with increased JAK2/STAT1 activity and enhanced expression of polycystin-1 C-terminal tail (PC1-CTT). Overexpression or blockage of STAT1 increased or decreased CFB expression and CFB promoter activity. Moreover, overexpression of PC1-CTT induced JAK2/STAT1 activation and CFB up-regulation in renal tubular epithelial cells. Furthermore, PC1-CTT over-expression increased human CFB promoter activity, whereas dominant negative STAT1 plasmids or mutation of putative STAT1 responsive elements decreased PC1-CTT induced CFB promoter activity. The effect of CFB on macrophage differentiation was tested on a mouse macrophage cell line. Bioactive CFB dose-dependently promoted macrophage M2 phenotype conversion. In addition, conditioned media from renal epithelial cells promoted macrophage M2 phenotype conversion which was blocked by STAT1 inhibition in a dose-dependent manner. Conditioned media from PC1-CTT transfected renal epithelial cells further promoted macrophage M2

phenotype conversion, which was suppressed by fludarabine or CFB antibody. In addition, we show that NF-κB acts downstream of PC1-CTT and may partly mediate PC1-CTT induced CFB expression. In conclusion, our study reveals possible mechanisms of CFB up-regulation in ADPKD and a novel role of PC1-CTT on ADPKD associated inflammation. Furthermore our study suggests that targeting STAT1 may be a new strategy to prevent inflammation in the kidney of patients with ADPKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the leading causes of end stage renal failure (21). Most cases of ADPKD (85%) are caused by mutations in the *PKD1* gene which encodes polycystin-1. Polycystin-1 is a large transmembrane protein containing a ~200 amino-acid cytoplasmic tail which can be cleaved into a full-length 30 kD peptide and a half-length 15 kD peptide upon mechanical stimulation (4, 18). The 30 kD peptide (PC1-CTT) is over-expressed in ADPKD and non-orthologous models of polycystic kidney disease (PKD) (18). PC1-CTT plays important roles in cell proliferation, apoptosis and polarity (2, 8, 18). Moreover over-expression of PC1-CTT induced renal cysts in the zebrafish model (9). As a co-transcriptional factor, PC1-CTT regulates multiple downstream signaling pathways such as the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (18). The JAK2/STAT1 pathway plays multiple roles in tumor genesis (1, 5). Among STAT family members, only STAT1, STAT3 and STAT6 have been studied in the field of PKD (19). STAT3 and STAT6 displayed proliferative and pro-cystic effects in animal models of PKD (10, 17). *In vitro* studies showed that STAT1 mediates polycystin-1 induced cell cycle arrest, however STAT1 promotes tumor growth mainly through its pro-inflammatory property, but this has not yet

been studied in PKD (1, 3, 5).

The role of inflammation in polycystic kidney disease has gained increasing attention in recent years (16). Inflammatory cells such as macrophages accumulate in polycystic kidney tissues in proximity to renal cysts and differentiate into an M2-phenotype. This could be triggered by undefined soluble factors which are secreted by the cystic epithelium (15).

We have previously shown that the alternative complement pathway is aberrantly activated in polycystic kidney disease, and that rosmarinic acid retards PKD progression. This is associated with decreased complement factor B (CFB) expression and reduced infiltration of macrophages (13, 14). However the underlying mechanisms of alternative complement pathway activation in polycystic kidneys in PKD remain unclear.

The purpose of this study was to explore the mechanism of CFB overexpression in PKD and to investigate whether polycystin-1 regulates CFB expression.

Materials and Methods

Reagents and Plasmids

The 712 bp human CFB promoter was sub-cloned into the pGL3-basic luciferase reporter vector. The site mutation of STAT1 response element on the CFB promoter plasmid was made using Fast mutagenesis system (Transgen, Beijing China). The 6-fold repeated GAS (5'-TTCCGGGAA-3') sequence and 6-fold repeated and mutated GAS (5'- CGTACGGCT-3') sequence were constructed on pGL3-basic vector. The vector pRL-TK was obtained from Promega (Madison, WI). PC1-CTT, DN-1-STAT1 (mutation at Y701F) and DN-2-STAT1 (mutation at S727A) plasmids were obtained from Addgene (18, 20). STAT1 was sub-cloned into pCDNA4/TO-myc-His vector.

Total STAT1 (#9172), p-JAK2 (#3776), and p-STAT1 (#7649) antibodies are from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody (sc-365062), PC1 C-terminal antibody (sc-10371), control rabbit IgG (sc-2027) are from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-myc from Abmart (#M20002, Shanghai, China). The CFB antibodies are from Proteintech (10170-1-AP) (Chicago, USA) and Santa Cruz (H95, sc-67141). Arginase-1 (ab60176) and iNOS (ab3523) antibodies are from

Abcam (Cambridge, UK). The STAT1 specific inhibitor fludarabine (S1491) and the NF-κB specific inhibitor QNZ were obtained from Selleckchem (Houston, TX, USA). Bioactive human CFB was purchased from Hycult biotech (Uden, Netherlands). IL-4 (#241-14) and IL-13 (#210-13) were from PeproTech (Rocky Hill, NJ, USA). INF-γ (Gibco, PMC4031) was bought from Invitrogen (USA).

Animal and Human Samples

Rat kidney tissues were collected from the Han: SPRD rat colony established in our animal facility from a litter which was obtained from Prof. Wuthrich (University of Zurich). Rats were kept according to local regulation and guidelines. Tissue samples from anonymous ADPKD patients and normal controls were obtained from Shanghai Changzheng Hospital. Informed consent was obtained from all participants.

Cell culture and transfection

Pkd1$^{+/-}$ and *Pkd1*$^{-/-}$ cells were obtained from Dr. Yiqiang Cai (Yale University) (6). Human immortalized renal epithelial cells (UCL93) were kindly provided by Prof. A.C. Ong (University of Sheffield, Sheffield, UK) (11). Cells were cultured to 70–80% confluence and transfected using

Ronfect reagent (Ronbio, Shanghai, China) according to the manufacturer's instructions, for 48 hr before protein extraction.

Protein Extraction and Western blot analysis

Snap frozen kidney tissue was homogenized in freshly made tissue protein extraction reagent (T-PER, Pierce Bioscience, Rockford, IL). Homogenates were centrifuged, and supernatants were stored at -80 °C.

To extract cell protein, cultured cells were washed and lysed in the same way. Lysates in SDS-sample buffer were boiled for 5 minutes at 95^{o}C and equal protein amounts were resolved by SDS-PAGE gels before transferring to a PVDF membrane. The membrane was probed with first antibody by overnight incubation at 4° C, and thereafter with a secondary antibody by incubation for 2 hr at room temperature. Proteins of interest were and then visualized by enhanced chemiluminescence detection reagents. The average densitometry values from three independent experiments were measured. Since the blot probed with polycstin-1 antibody had a high background when using the standard protocol, we used a lower dilution of the first antibody (1:4000) and the second antibody (1:5000). In addition, the blot was incubated with the second antibody for only 2 hr at 4° C before

visualization.

Immunohistochemical analysis

Immunhistochemistry was performed on paraffin sections of formalin-fixed tissues (3 μm thick). Sections were incubated overnight at 4 ^{o}C with primary antibody against p-JAK2 (1:100, Abcam, ab32101), p-STAT1 (1:100, Affinity, AF3299), CFB antibody (1:100, Proteintech, 10170-1-AP) and PC1 C-terminal antibody (1:200, Santa cruz, sc-10371). Signals were detected using Dako EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse (K5007). Micrographs were made randomly using the x400 magnification of the light microscope.

Dual-Luciferase Reporter Assay

UCL93 cells were cultured to 70–80% confluency in 48-well plate and using Ronfect transfection reagent according to manufacturer's instructions, using 25 ng reporter construct, 25 ng pRL-TK plasmid and 50 ng expression plasmid, then cultured for 24 hr in starve medium. A dual-luciferase reporter assay system (Promega) was used for measurements of firefly and Renilla luciferase activities. At least three independent transfections were performed, and all analyses were performed in quadruplicate. The average values in

each experiment were measured.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted using the Beyotime-ChIP Assay Kit, according to the manufacturer's protocol (P2078, Beyotime, Nanjing China). Immunoprecipitation was performed with the anti-total STAT1 antibody. Two PCR primer pairs were designed with one containing a putative STAT-binding site, the other primer pair spans -712/-373 region representing a non-specific region of CFB promoter. PCR products were analyzed by 2.5 % agarose/ethidium bromide gel electrophoresis.

***In vitro* macrophage differentiation assay**

UCL93 cells were cultured in DMEM/F12 medium containing 2% FBS in 6-well plate. Medium was changed 16hr after transfection or fludarabine treatment, then cells were cultured for another 24hr with starvation medium. In some experiments, the STAT1 inhibitor fludarabine was added 6hr after transfection and medium was changed 16hr after transfection. Cell free supernatants was named conditioned medium (CM) and stored in aliquots at – 80 ^{o}C.

Approximately 1×10^6 RAW264.7 cells were cultured in 2% FBS medium in 6-well plate overnight before experiments. The CM from UCL93 cells was used to treat mouse RAW264.7 cells. In some experiments, bioactive human CFB or CFB antibodies were added in un-conditioned medium or conditioned medium as indicated elsewhere. As positive controls, 100 μg/ml IL-4/IL-13 or 100 ng/ml INF-γ was added into un-conditioned medium to induce macrophage differentiation. After 24hr of treatment, protein was extracted and probed for iNOS and arginase-1 using Western blot as described above.

Real-Time PCR

Total RNA was extracted using RNase mini kit (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed. The primer sequences were:

Arg1, forward, 5'-CTCCAAGCCAAAGTCCTTAGAG; reverse, 5'-GGAGCTGTCATTAGGGACATC; iNOS, forward, 5'-GTTCTCAGCCCAACAATACAAGA; reverse, 5'-GTGGACGGGTCGATGTCAC; Mrc1, forward, 5'-CTCTGTTCAGCTATTGGACGC; reverse, 5'-CGGAATTTCTGGGATTCAGCTTC; GAPDH, forward, 5'-AGAACATCATCCCTGCATCC; reverse, 5'-

ATACCAGGAAATGAGCTTGAC; real-time PCR was performed using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and Rotor-Gene-3000A real-time PCR system (Corbett, Sydney, Australia), according to the manufacturer's protocol. In brief, the PCR amplification reaction mixture (25 μl) contained 1 μl cDNA, 0.4 mM sense and antisense primer, and 12.5 μl SYBR Green I. After initial denaturation at 95° C for 1 min, the reaction was cycled 45 times. Each cycle consisted of denaturation at 95 °C for 15 s, primer annealing and extension at 60 °C for 31 s. The results are given as relative expression of Arg1, iNOS, Mrc1 normalized to the expression of GAPDH. Real-time PCR was done in triplicate for each experiment, and the average values were measured. Each experiment was repeated three times.

Statistical Analysis

Statistical analyses were performed by unpaired t-test or one-way ANOVA with the Newman-Keuls post hoc test using GraphPad Prism version 5.0 (GraphPad, San Diego, CA). All data are expressed as means ± SD, and $P < 0.05$ was considered as statistically significant.

Results:

1. CFB overexpression correlates with PC1-CTT overexpression and JAK/STAT1 activation in polycystic kidney tissues

Figure 1A and 1B show that CFB was up-regulated in human and rat cystic kidney tissues which was correlated with increased p-JAK2, p-STAT1 and PC1 C-terminal tail (PC1-CTT).

Immunohistochemistry was performed on rat kidney tissues to confirm the finding by Western blot. Figure 1C shows that signals for p-JAK2, p-STAT1, CFB and PC1-CTT were enhanced in Cy/+ cystic kidneys, where a similar expression pattern was found for these proteins. All these proteins were strongly stained in dilated tubules and small cysts but were weakly stained in large cysts.

Since the specificity of the commercially available polycystin-1 antibody for PC1-CTT has not been reported, we performed two experiments to test its specificity. Figure 2A shows that PC1-CTT can be detected by this polycystin-1 antibody in *Pkd1*$^{+/-}$ cells but not *Pkd1*$^{-/-}$ cells. Moreover, overexpression of exogenous PC1-CTT could be detected by the polycystin-1 antibody and also by the myc-tag antibody (Figure 2B).

2. STAT1 regulates CFB expression

To determine whether STAT1 regulates CFB expression in renal epithelial cells, we over-expressed STAT1 plasmids in UCL93 immortalized human renal epithelial cells. Figure 3A shows that overexpression of STAT1 increased CFB expression as compared to control cells which were transfected with empty vectors. Moreover, the STAT1 inhibitor fludarabine dose-dependently reduced CFB expression which was correlated with decreased p-STAT1 levels (Figure 3B).

The putative STAT1-binding site TTCCGAGAA was identified in the promoter sequence of the human CFB gene, at position -219 to -210 upstream of translation start site (Figure 4A). STAT1 gene, CFB promoter (CFB-pr) and renilla were co-transfected in renal epithelial cells and were assayed for luciferase activity. Renilla values obtained were used for normalization of CFB promoter activity. Empty vector, CFB-pr and renilla co-transfected cells were used as control. STAT1 transfection leads to a 6 fold increase in promoter activity (Figure 4B). Luciferase activity did not increase in cells transfected with STAT1 binding site mutated CFB promoter (CFB-pr-Mut) (Figure 4B). It is known that the gamma interferon activation site (GAS) sequence contains a STAT1 binding site (7). Thus we designed a reporter gene containing 6 repeated GAS sequences (GAS

5’-TTCCGGGAA-3’) and another reporter gene containing 6 mutated and repeated GAS sequences (GAS mutation 5’-CGTACGGCT). In Figure 4C, we show that CFB reporter gene and GAS reporter gene have similar responses to STAT1 stimulation, and that the GAS mutation abolished the response of GAS reporter gene to STAT1 stimulation.

To confirm the direct association of the STAT1 protein with human CFB promoter, we performed chromatin immunoprecipitation (ChIP) assays. The PCR primer pair 1 was designed to flank the potential STAT1 site (-382 to -138), and we observed that the STAT1 protein level was increased in this region in STAT1 over-expressed 293T cells compared to empty vector transfected control cells. The second selected region (−712 to −373) was amplified by the primer pair 2, which does not contain a STAT1 response element and represents a non-specific region in the CFB promoter (Figure 4D). No STAT1 protein was enriched in the second region.

3. STAT1 mediates PC1-CTT induced CFB expression

To test whether PC1 C-terminal tail (PC1-CTT) regulates CFB expression, a soluble PC1-CTT was over-expressed in UCL93 renal epithelial cells. PC1-CTT increased CFB expression which was correlated with enhanced

phosphorylation of JAK2 and STAT1 in renal epithelial cells (Figure 5A).

We next tested whether the PC1-CTT induced expression of CFB requires STAT1, PC1-CTT transfected renal epithelial cells were treated with fludarabine. The STAT1 inhibitor fludarabine suppressed PC1-CTT induced CFB overexpression and p-STAT1 up-regulation in renal epithelial cells (Figure 5B).

Mutation of the STAT1 binding site significantly down-regulated the PC1-CTT induced CFB promoter activity in UCL93 cells by 29% (Figure 6A), which suggests that the STAT1 response element at −219 to −210 is important for PC1-CTT mediated transactivation of CFB promoter.

To further investigate the mechanism of STAT1 regulation of CFB expression, PC1-CTT plasmid was co-transfected with two different dominant-negative (DN) STAT1 plasmids (DN1-STAT1 with Y701F mutation or DN2-STAT1 with S727A mutation). DN1-STAT1 and DN2-STAT1 significantly suppressed PC1-CTT induced CFB promoter activity as compared with PC1-CTT and empty vector co-transfected cells (Figure 6B). Furthermore, we showed that CFB reporter gene and GAS reporter gene have similarly increased responses to PC1-CTT stimulation, and that the GAS mutation abolished the response of GAS reporter gene to PC1-CTT stimulation (Figure 6C). Taken together our data indicate that

315 PC1-CTT is capable of activating CFB transcription through the promoter
316 element in the region −219 to −210 which contains the STAT1 binding site.
317
318 **4. PC1-CTT stimulates macrophage differentiation through STAT1 and**
319 **CFB**
320 To study whether CFB triggers macrophage M2 phenotype differentiation,
321 bioactive human CFB was used to treat RAW264.7 mouse macrophage cells.
322 Figure 7A shows that human CFB dose-dependently up-regulated the M2
323 marker arginase-1 (Arg1) in mouse macrophages from 30 ng/ml to 100
324 ng/ml, and the expression of M1 phenotype marker iNOS was not changed
325 by CFB stimulation. RT-PCR was performed to further analyze macrophage
326 differentiation. Figure 8A shows that the M2 marker Arg1 but not Mrc1 was
327 increased by CFB treatment. However, CFB induced M2 macrophages had
328 lower Arg1 expression as compared with typical M2 macrophages induced
329 by IL4/IL13. Again, the mRNA expression of the M1 markers iNOS or TNF-
330 α was not up-regulated by CFB treatment (Figure 8B). Moreover, CFB
331 antibody was included in conditioned medium that was generated from renal
332 epithelial cells transfected with PC1-CTT (PC1-CTT-CM). Treatment of
333 RAW264.7 cells with the conditioned media from PC1-CTT over-
334 expressing renal epithelial cells markedly increased the protein level of

arginase-1 but not iNOS, whereas CFB antibody neutralization in PC1-CTT-CM suppressed arginase-1 expression in Raw 264.7 macrophage cells dose-dependently (Figure 7B). RT-PCR analysis showed that PC1-CTT conditioned media induced significantly higher expression of the M2 markers Arg1 and Mrc1, but did not induce M1 macrophage differentiation (Figure 8C and 8D). Interestingly, PC1-CTT induced similar level of Arg1 expression but less Mrc1 expression as compared with IL4/IL13 induced M2 macrophages (Figure 8C).

To explore the role of STAT1 in renal epithelial cell induced M2 macrophage differentiation, we generated conditioned medium from renal epithelial cells which were treated with STAT1 specific inhibitor fludarabine. Figure 7C shows that STAT1 inhibition in renal epithelial cells reduced arginase-1 expression by macrophages in a dose-dependent manner. Moreover, STAT1 inhibition by fludarabine in renal epithelial cells suppressed arginase-1 expression induced by PC1-CTT-CM (Figure 7D).

Taken together our data suggest that PC1-CTT induced macrophage activation into an M2 phenotype is mediated by STAT1 and CFB.

5. NF-κB partly mediates PC1-CTT induced CFB expression

To test whether the NF-κB pathway partly mediates PC1-CTT induced CFB

355 expression, we first treat renal epithelial cells with the NF-κB inhibitor
356 quinazoline (6-amino-4-(4-phenoxypheny lethylamino; QNZ). Figure 9A
357 shows that QNZ dose-dependently down-regulated CFB expression which
358 was correlated with down-regulation of p50/p65 NF-κB. Second, PC1-CTT
359 induced up-regulation of p50/p65 NF-κB and CFB, and QNZ
360 down-regulated p50/p65 and CFB expression (Figure 9B). Third, we found
361 that CFB promoter activity triggered by PC1-CTT was reduced by NF-κB
362 inhibitor QNZ (Figure 9C).
363
364

Discussion:

We previously showed that CFB is up-regulated and plays an important role in human ADPKD and in the Han:SPRD rat model of PKD, however the mechanism of CFB overexpression in PKD is not known (13, 14). Huang *et al.* showed that STAT1 mediates IFN-γ induced mouse CFB expression through direct interaction with murine CFB promoter (7). Therefore we hypothesized that STAT1 regulates CFB expression in PKD. In this study, we proved this hypothesis by showing that 1) STAT1 activation correlated with CFB expression in rat and human cystic kidney tissues; 2) over-expression of STAT1 in renal epithelial cells increased CFB expression; 3) STAT1 inhibition by fludarabine reduced CFB expression; 4) STAT1 over-expression enhanced CFB promoter activity, and a mutation of the STAT1 binding site on CFB promoter abolished this effect; 5) STAT1 interacted with human CFB promoter.

It is known that the cytoplasmic tail of polycystin-1 can be cleaved into a 30 KD soluble peptide (PC1-CTT) which can function as a co-transcriptional factor entering the cell nucleus and regulating down-stream gene expression (2, 4, 18). Moreover it was shown that PC1-CTT increases STAT1 transcriptional activity (18). Therefore we hypothesized that PC1-CTT up-regulates CFB expression through STAT1 in PKD. Here we showed that

385 the PC1-CTT over-expression correlated with JAK2 and STAT1 activation in
386 human and rat polycystic kidneys where CFB was abnormally up-regulated.
387 Secondly, PC1-CTT over-expression in renal epithelial cells activated
388 JAK2/STAT1 pathway and led to up-regulation of CFB. Finally, STAT1
389 inhibition abolished PC1-CTT induced CFB expression and CFB promoter
390 activation.
391 Interestingly, we found that mutation of the STAT1 binding site only partly
392 abolished PC1-CTT triggered CFB promoter activity, whereas the mutant
393 promoter completely blocked STAT1 induced CFB promoter activation.
394 These data suggest that a fraction of the PC1-CTT effect on CFB promoter is
395 independent of STAT1. It has been shown that NF-κB could regulate CFB
396 expression, and that the NF-κB pathway is activated in cystic kidneys (12,
397 16). We therefore postulated that NF-κB could partly mediate PC1-CTT
398 induced CFB expression. We proved this by showing that 1) NF-κB inhibitor
399 QNZ down-regulated CFB expression in renal epithelial cells; 2) the reporter
400 activity driven by PC1-CTT can be reduced by NF-κB inhibitor QNZ; 3)
401 PC1-CTT induced up-regulation of p50/p65 NF-κB and CFB, and moreover
402 QNZ down-regulated p50/p65 and CFB expression.
403 The role of STAT1 in PKD is not completely studied. It has been shown that
404 polycystin-1 induces cell cycle arrest through STAT1 activation, suggesting

405 an anti-proliferative and therefore a protective role of STAT1 in PKD (3).
406 However, our data showed that STAT1 mediates PC1-CTT induced CFB
407 expression and macrophage differentiation *in vitro*. We further confirmed the
408 correlation of STAT1 activation and CFB expression in human and rat
409 polycystic kidneys. In liver disease, IFN-γ activation of STAT1 induces
410 apoptosis-associated liver inflammation (5). Interestingly, in a
411 non-orthologous PKD mouse model, where PC1 tail is over-expressed,
412 IFN-γ challenge resulted in a strong STAT1 activation and increased
413 apoptosis level. These data suggests a pro-inflammatory role of STAT1 in
414 PKD. Therefore an *in vivo* study should be performed with STAT1 inhibitor
415 or by STAT1 gene depletion to confirm its role in PKD.
416 Macrophages which have infiltrated the cystic kidneys present with a special
417 M2 phenotype which is characterized by highly increased arginase-1
418 expression and slightly increased Mrc1 expression (15). Our study suggests
419 that PC1-CTT and CFB participate in cystic M2 macrophage differentiation.
420 First, with different M1 or M2 markers and with two different approaches,
421 we showed that PC1-CTT or CFB promoted macrophage M2 but not M1
422 phenotype conversion. Second, PC1-CTT or CFB stimulated arginase-1
423 expression but not or to a lesser degree Mrc1 expression which is consistent
424 to the cystic M2 phenotype. Interestingly, compared with IL4/IL13 induced

conventional M2 macrophages, PC1-CTT stimulated similar levels of argniase-1 expression but decreased levels of Mrc1 expression, whereas CFB triggered macrophages had much less arignase-1 expression and Mrc1 expression. These data suggest that there are other un-defined factors, different from CFB that participate in PC1-CTT induced macrophage differentiation.

The M2 macrophages that infiltrate the cystic kidney may promote cyst expansion by directly or indirectly stimulating cyst epithelial cell proliferation (15, 16). We showed that conditioned media derived from STAT1-inhibited renal epithelial cells retarded macrophage M2 differentiation. Therefore STAT1 may also play a proliferative role in ADPKD through activating macrophage.

The overexpression of PC1-CTT is observed in human ADPKD and also in non-orthologous animal models of PKD (18). Ureteral ligation also induced up-regulation of PC1-CTT in non-mutated kidney suggesting that alteration of tubular fluid flow triggers the cleavage of cytoplasmic tail from the mechanical sensor polycystin-1 (4). In this study we found a novel function of PC1-CTT in PKD inflammation, namely increased CFB expression in renal epithelial cells and modulated macrophage differentiation. Interestingly, we found PC1-CTT and CFB strongly expressed in small cysts. It has been

shown that in ADPKD kidney tissues, macrophages localize mostly adjacent to renal cysts or even infiltrate small cysts (15). Thus it could be speculated that the change of tubular fluid flow by expanding renal cysts initiates inflammation through increasing cleavage of polycystin-1 and this might lead to the genesis of new cysts.

In summary, our study showed that CFB over-expression in PKD is caused at least in part by PC1-CTT induced STAT1 activation, and by activation of the NF-κB pathway. STAT1 could be a new target to prevent PKD inflammation.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure Legends

Figure 1

PC1-CTT, p-JAK2, p-STAT1 and CFB expression in PKD. A. Phosphorylated JAK2, total JAK2, phosphorylated STAT1, total STAT1, CFB and PC1-CTT in control and human ADPKD kidneys were analysed by Western blot, and then quantified by densitometry. Representative Western blot is shown, and results represent an average of three independent. B. Phosphorylated JAK2, total JAK2, phosphorylated STAT1, total STAT1, CFB and PC1-CTT in +/+ and Cy/+ Han:SPRD rat kidneys were analysed by Western blot, and then quantified by densitometry. Representative Western blot is shown, and results represent an average of three independent. C. Immunohistochemistry staining of phosphorylated JAK2, phosphorylated STAT1, CFB and PC1-CTT in Han:SPRD rat kidneys are shown. One representative of three independent experiments is shown, bars = 40μm. Not significant (NS), *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

Specificity of polycystin-1 antibody for PC1-CTT. The expression of PC1-CTT in *Pkd1*$^{+/-}$, *Pkd1*$^{-/-}$, UCL93 control and PC1-CTT overexpressing

549 UCL93 cells. PC1-CTT was detected by the polycystin-1 (PC1) or myc
550 antibody. Blots were quantified by densitometry, and the average
551 densitometry values from three independent experiments is shown. **P <
552 0.01, ***P < 0.001. One representative of three independent experiments is
553 shown.
554
555 **Figure 3**
556 The effect of STAT1 on CFB expression. A. CFB expression in STAT1
557 over-expressed UCL93 renal epithelial cells 48hr after transfection. Blots
558 were quantified by densitometry, and the average densitometry values from
559 three independent experiments is shown. B. CFB expression in renal
560 epithelial cells which were treated with the STAT1 inhibitor fludarabine for
561 48hr. Blots were quantified by densitometry, and the average densitometry
562 values from three independent experiments is shown. Not significant (NS),
563 *P < 0.05, **P < 0.01, ***P < 0.001. One representative Western blot of
564 three independent experiments is shown.
565
566 **Figure 4**
567 A. The representation of CFB promoter with putative STAT binding site
568 (−219/-210). B. Luciferase activity of CFB promoter (CFB-Pr) or mutated

CFB promoter (CFB-Pr-Mut) were measured in control or STAT1 transfected UCL93 cells. C. Luciferase activity of CFB promoter (CFB-Pr), GAS promoter (GAS-Pr) or mutated GAS promoter (GAS-Pr-Mut) were measured in control or STAT1 transfected UCL93 cells. D. ChIP assay was conducted on 293T cells transfected with control or STAT1. Two different regions (−700/-350, -400/-100) were amplified by PCR after chromatin immunoprecipitation using anti- STAT1 antibody. Not significant (NS), **P < 0.01. One representative of three independent experiments is shown.

Figure 5

The effect of PC1-CTT on JAK2/STAT1 activation and CFB expression. A. JAK2/STAT1 activation and CFB expression in UCL93 renal epithelial cell lines after 48hr PC1-CTT transfection. Blots were quantified by densitometry, and the average densitometry values from three independent experiments is shown. B. PC1-CTT was transfected in renal epithelial cells and followed by 24hr fludarabine treatment, CFB expression was analysed by Western blot and then quantified by densitometry. The average densitometry values from three independent experiments is shown. Not significant (NS), *P<0.05, **P < 0.01, ***P < 0.001. One representative Western blot of three independent experiments is shown.

589

590 **Figure 6**

591 A. Luciferase activity of CFB promoter (CFB-Pr) or mutated CFB promoter
592 (CFB-Pr-Mut) were measured in control or PC1-CTT transfected renal
593 epithelial cells. B. Co-transfection of renal epithelial cells with DN-STAT1
594 (DN1-STAT1 or DN2-STAT1) attenuated PC1-CTT induced CFB promoter
595 activity. C. Luciferase activity of CFB promoter (CFB-Pr), GAS promoter
596 (GAS-Pr) or mutated GAS promoter (GAS-Pr-Mut) were measured in
597 control or PC1-CTT transfected UCL93 cells. Not significant (NS), *$P<0.05$,
598 **$P < 0.01$. One representative of three independent experiments is shown.

599

600 **Figure 7**

601 PC1-CTT promotes RAW264.7 macrophages toward M2 phenotype
602 differentiation through STAT1 and CFB. A. Bioactive human CFB was
603 added into un-conditioned media to treat macrophages. The expression of
604 macrophage M1 phenotype marker iNOS and M2 phenotype marker
605 arginase-1 were examined by Western blot. Blots were quantified by
606 densitometry, and the average densitometry values from three independent
607 experiments is shown. B. Conditioned media were prepared from control
608 (Control-CM) or PC1-CTT (PC1-CTT-CM) transfected renal epithelial cells.

Isotype IgG or CFB antibody was added to treat macrophages. C. Conditioned media were generated from renal epithelial cells treated with fludarabine and used to treat macrophages for 24hr. D. PC1-CTT was transfected in renal epithelial cells and followed by fludarabine treatment, and conditioned media were collected to treat macrophages. *P < 0.05, **P < 0.01, ***P < 0.001. One representative of three independent experiments is shown.

Figure 8

PC1-CTT promotes differentiation of RAW264.7 macrophages towards M2 phenotype through CFB. A and B. Bioactive human CFB was added into un-conditioned media to treat macrophages. IFN- γ or IL4/IL13 was used as a positive control to induce M1 or M2 macrophage phenotype, respectively. The expression of macrophage M1 phenotype markers iNOS or TNF-α, and M2 phenotype markers arginase-1 (Arg1) or Mrc1 were examined by RT-PCR. C and D. Conditioned media were collected from control (Control-CM) or PC1-CTT (PC1-CTT-CM) transfected renal epithelial cells to treat macrophages, then M1/M2 markers were analysed by RT-PCR. Not significant (NS), *P < 0.05, ***P < 0.001. One representative of three independent experiments is shown.

629

630 **Figure 9**

631 NF-κB mediates PC1-CTT induced CFB expression. A. NF-κB (p50 and p65)
632 and CFB expression in renal epithelial cells which were treated with the
633 NF-κB inhibitor QNZ for 48 hr. B. PC1-CTT was transfected in renal
634 epithelial cells and followed by 24 hr QNZ treatment, NF-κB (p50 and p65)
635 and CFB expression were analysed by Western blot. Blots were quantified
636 by densitometry, and the average densitometry values from three
637 independent experiments is shown. C. Luciferase activity of CFB promoter
638 (CFB-Pr) was measured in control or PC1-CTT transfected renal epithelial
639 cells or QNZ treated PC1-CTT transfected cells. *$P < 0.05$, **$P < 0.01$, ***P
640 < 0.001. One representative of three independent experiments is shown.

641

Figure 1

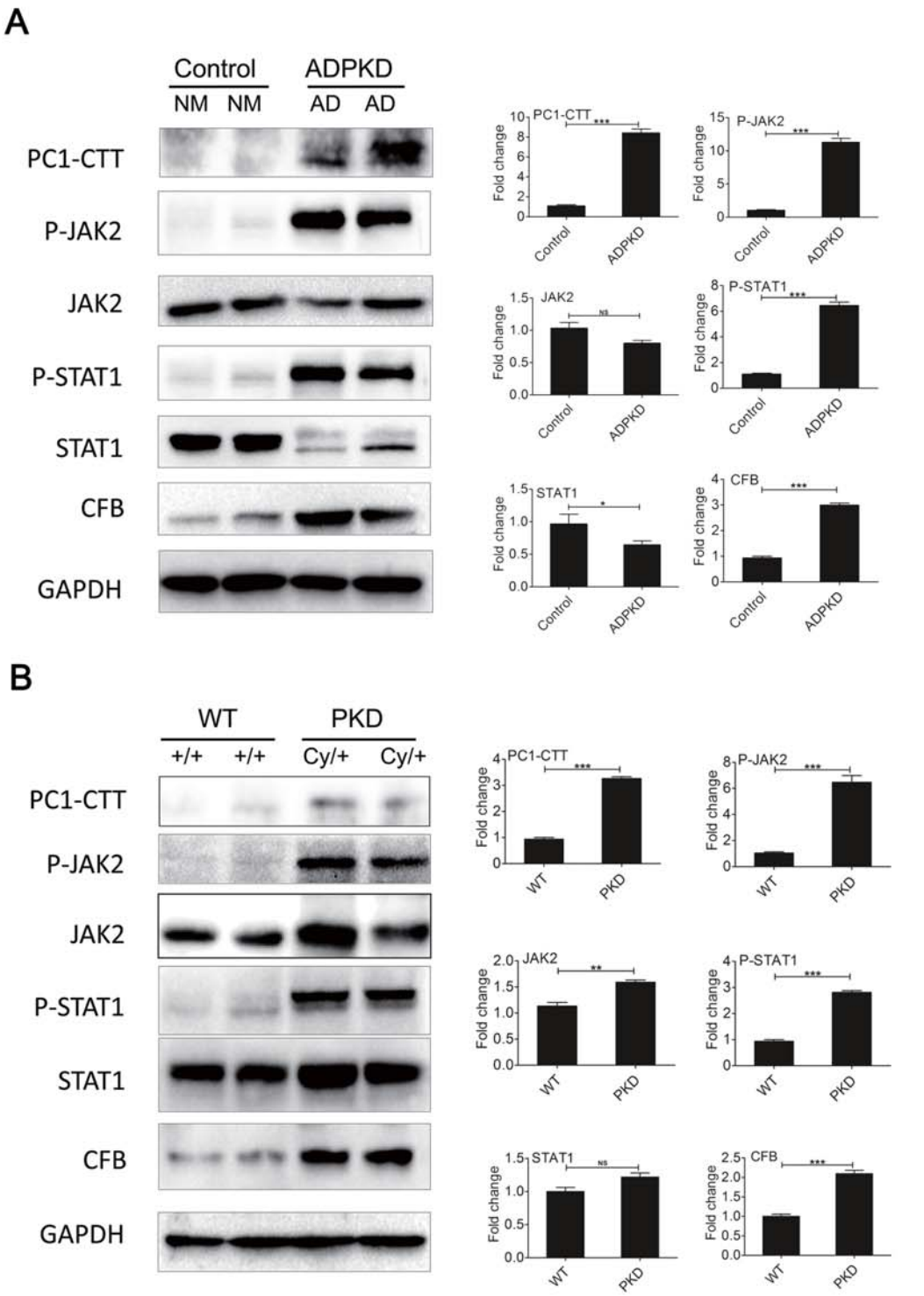

A
Control
ADPKD
NM NM
AD AD
PC1-CTT
P-JAK2
JAK2
P-STAT1
STAT1
CFB
GAPDH
Fold change
Control
ADPKD
B
WT
PKD
+/+ +/+
Cy/+ Cy/+
WT
PKD
C
p-JAK2
p-STAT1
CFB
PC1-CTT
+/+
Cy/+

Figure 2

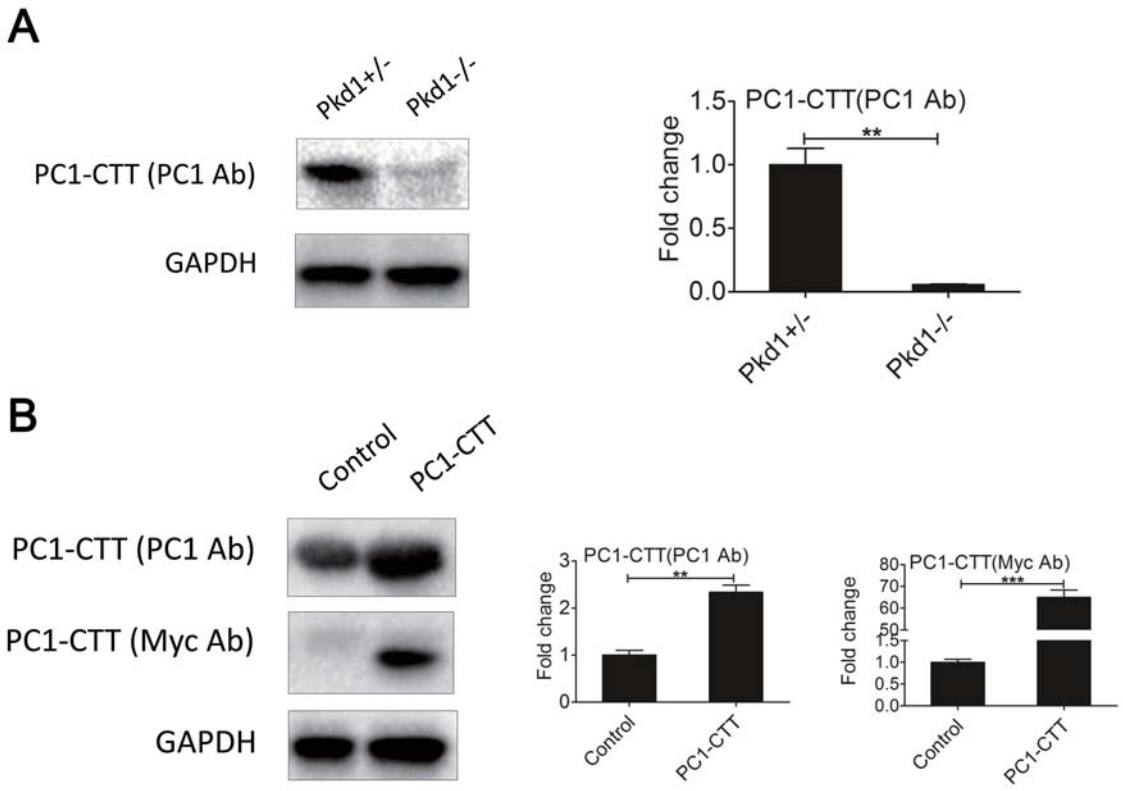

A
Pkd1+/-
Pkd1-/-
PC1-CTT (PC1 Ab)
GAPDH
PC1-CTT(PC1 Ab)
Fold change
1.5
1.0
0.5
0.0
**
Pkd1+/-
Pkd1-/-
B
Control
PC1-CTT
PC1-CTT (PC1 Ab)
PC1-CTT (Myc Ab)
GAPDH
PC1-CTT(PC1 Ab)
Fold change
3
2
1
0
**
Control
PC1-CTT
PC1-CTT(Myc Ab)
Fold change
80
70
60
50
1.5
1.0
0.5
0.0
***
Control
PC1-CTT

Figure 3

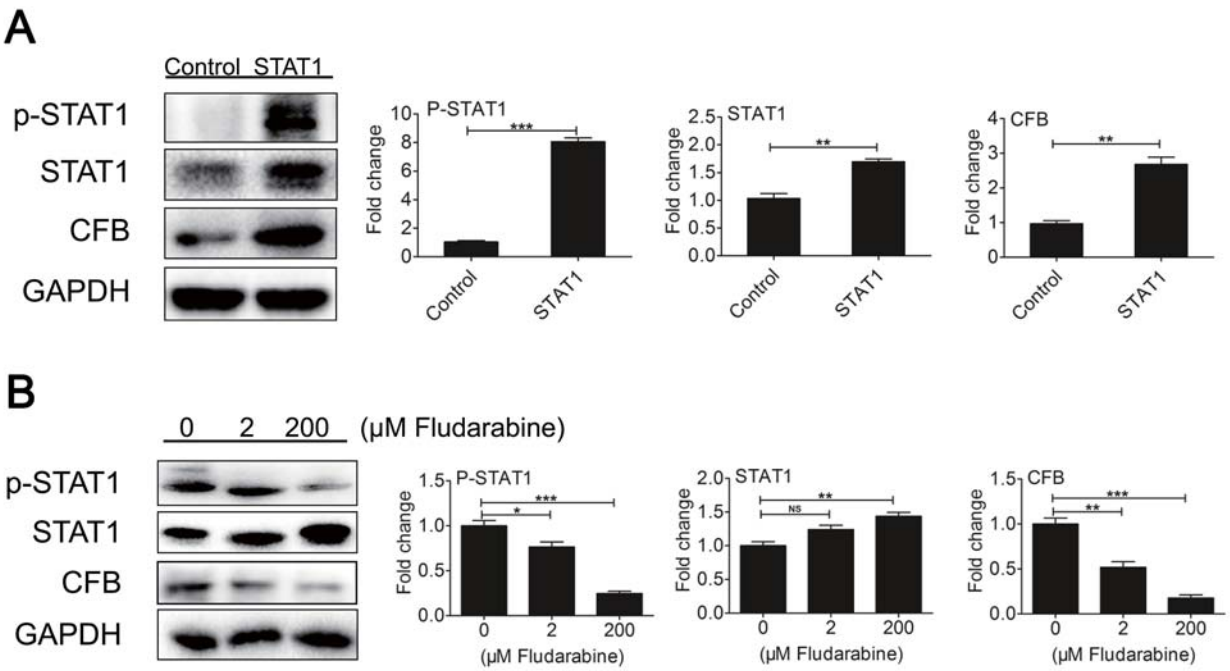

A
Control STAT1
p-STAT1
STAT1
CFB
GAPDH
P-STAT1
Fold change
Control
STAT1
STAT1
CFB
B
0 2 200 (μM Fludarabine)
NS
(μM Fludarabine)

Figure 4

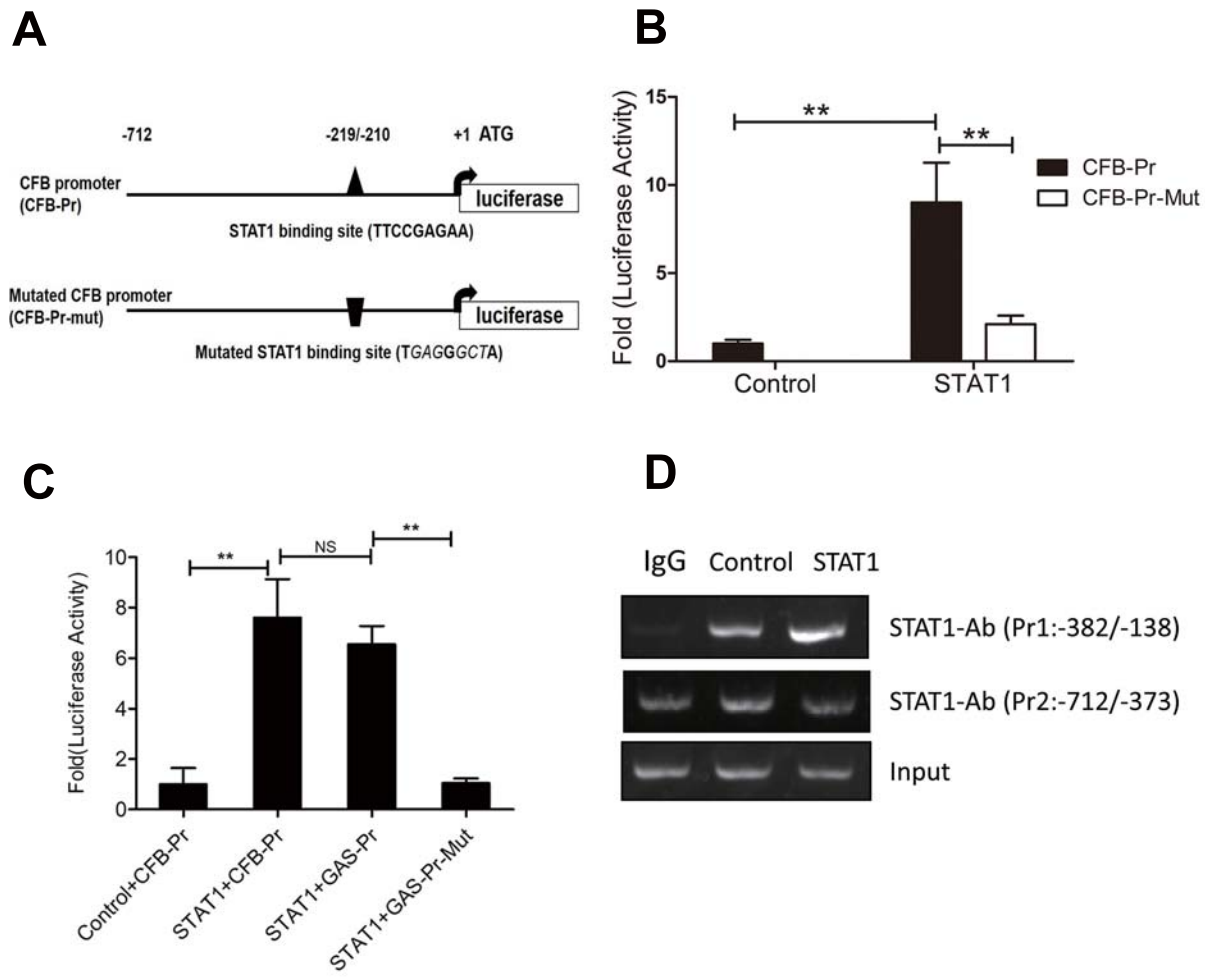

A
-712
-219/-210
+1 ATG
CFB promoter
(CFB-Pr)
luciferase
STAT1 binding site (TTCCGAGAA)
Mutated CFB promoter
(CFB-Pr-mut)
luciferase
Mutated STAT1 binding site (TGAGGGCTA)
B
Fold (Luciferase Activity)
15
10
5
0
**
**
CFB-Pr
CFB-Pr-Mut
Control
STAT1
C
Fold(Luciferase Activity)
10
8
6
4
2
0
**
NS
**
Control+CFB-Pr
STAT1+CFB-Pr
STAT1+GAS-Pr
STAT1+GAS-Pr-Mut
D
IgG
Control
STAT1
STAT1-Ab (Pr1:-382/-138)
STAT1-Ab (Pr2:-712/-373)
Input

Figure 5

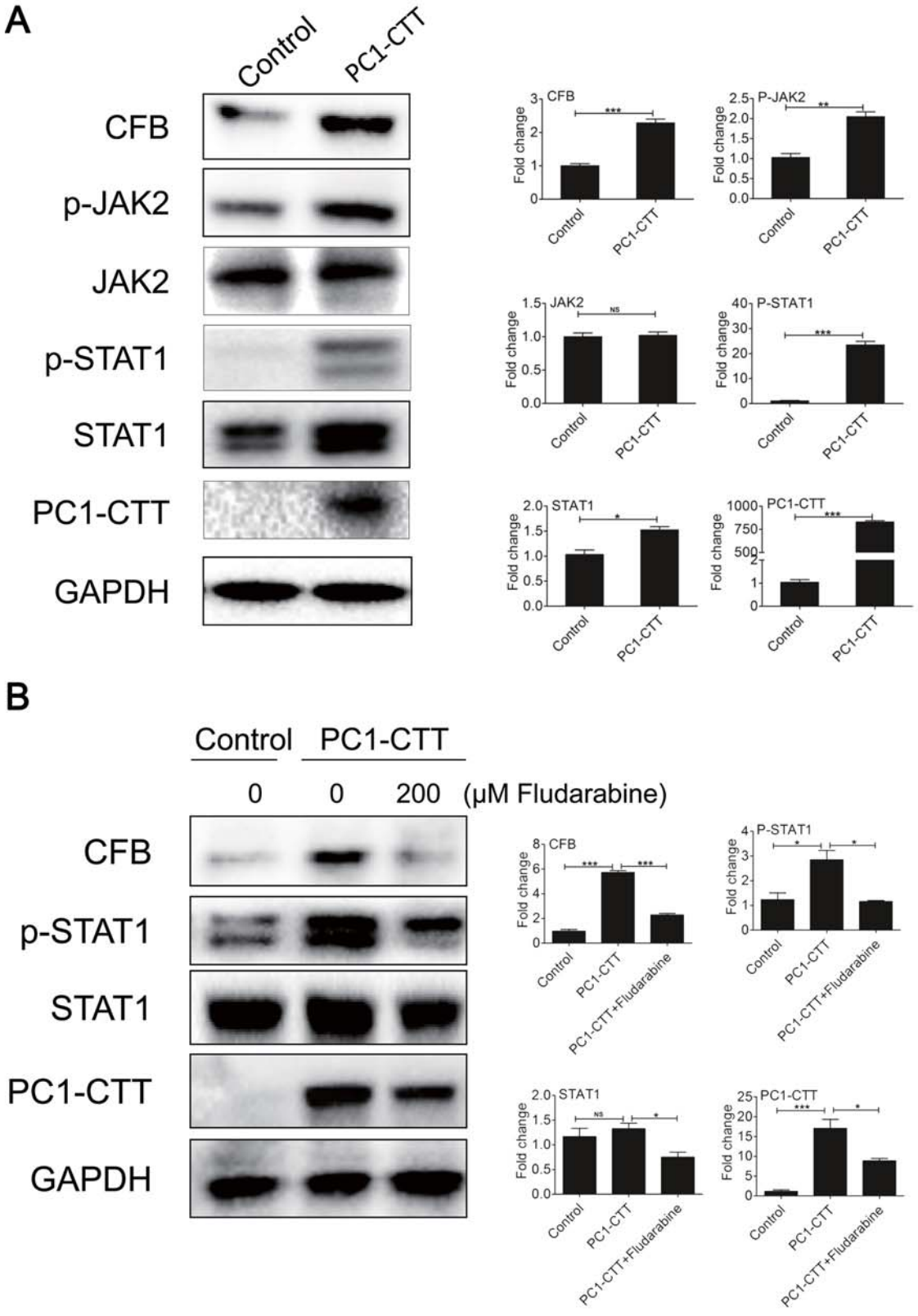

A
Control
PC1-CTT
CFB
p-JAK2
JAK2
p-STAT1
STAT1
PC1-CTT
GAPDH
CFB
P-JAK2
JAK2
P-STAT1
STAT1
PC1-CTT
Fold change
Control
PC1-CTT
B
Control
PC1-CTT
0
0
200
(μM Fludarabine)
CFB
p-STAT1
STAT1
PC1-CTT
GAPDH
P-STAT1
STAT1
PC1-CTT
Control
PC1-CTT
PC1-CTT+Fludarabine

Figure 6

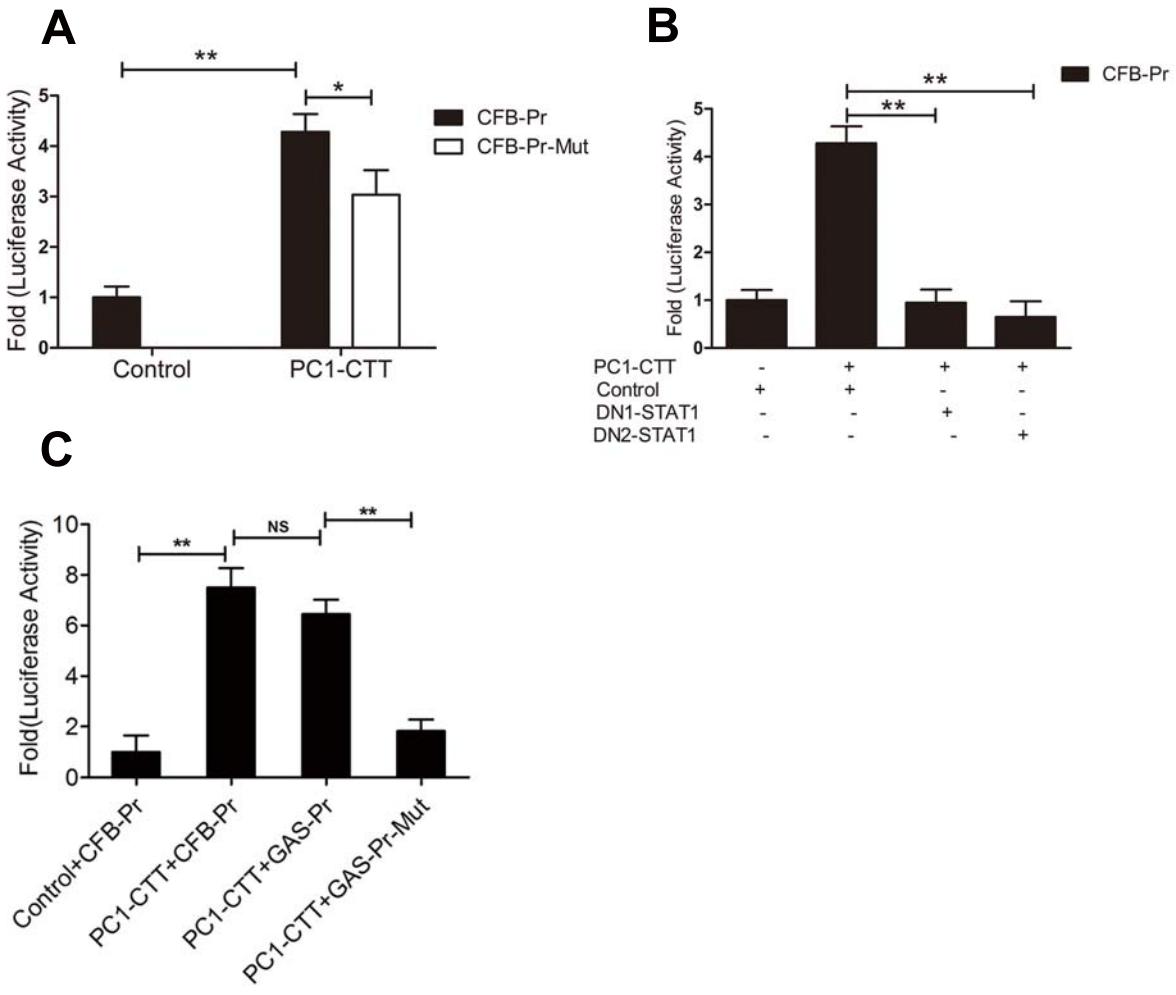

A
**
*
CFB-Pr
CFB-Pr-Mut
Fold (Luciferase Activity)
0
1
2
3
4
5
Control
PC1-CTT
B
**
**
CFB-Pr
Fold (Luciferase Activity)
PC1-CTT - + + +
Control + + - -
DN1-STAT1 - - + -
DN2-STAT1 - - - +
C
**
NS
**
Fold(Luciferase Activity)
0
2
4
6
8
10
Control+CFB-Pr
PC1-CTT+CFB-Pr
PC1-CTT+GAS-Pr
PC1-CTT+GAS-Pr-Mut

Figure 7

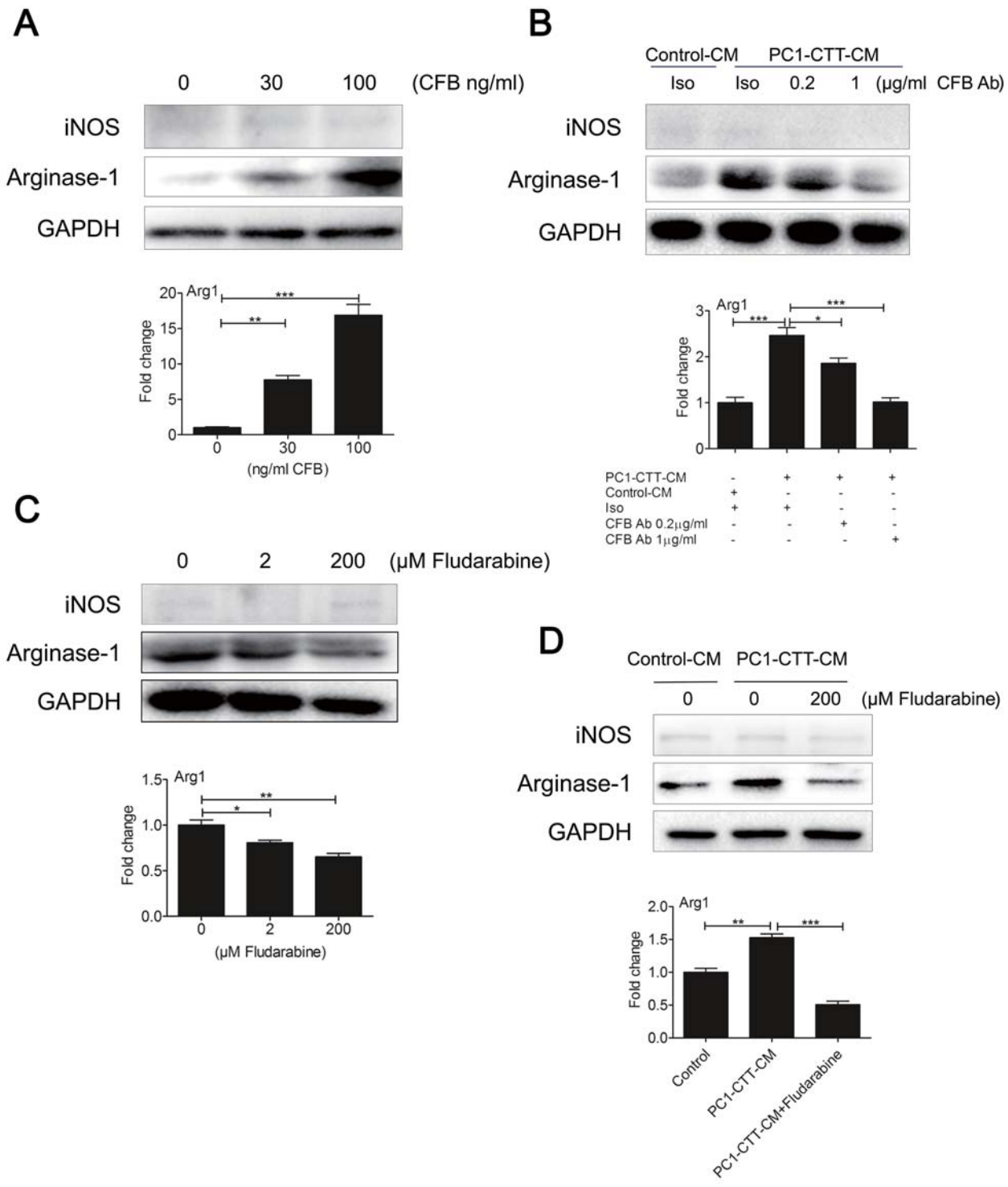

A
0
30
100
(CFB ng/ml)
iNOS
Arginase-1
GAPDH
Arg1
Fold change
(ng/ml CFB)
B
Control-CM
PC1-CTT-CM
Iso
Iso
0.2
1
(μg/ml CFB Ab)
iNOS
Arginase-1
GAPDH
Arg1
Fold change
PC1-CTT-CM
Control-CM
Iso
CFB Ab 0.2μg/ml
CFB Ab 1μg/ml
C
0
2
200
(μM Fludarabine)
iNOS
Arginase-1
GAPDH
Arg1
Fold change
(μM Fludarabine)
D
Control-CM
PC1-CTT-CM
0
0
200
(μM Fludarabine)
iNOS
Arginase-1
GAPDH
Arg1
Fold change
Control
PC1-CTT-CM
PC1-CTT-CM+Fludarabine

Figure 8

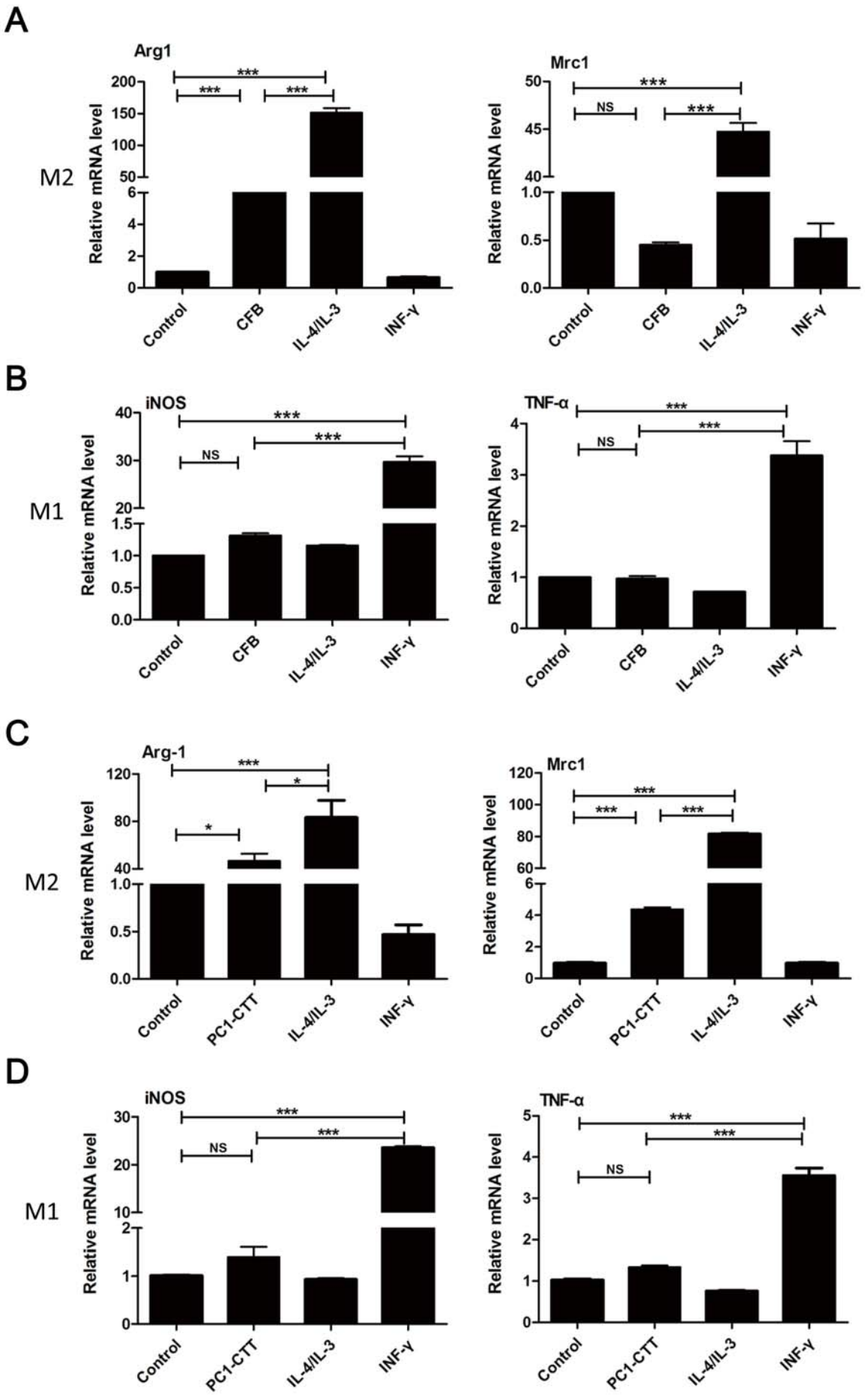
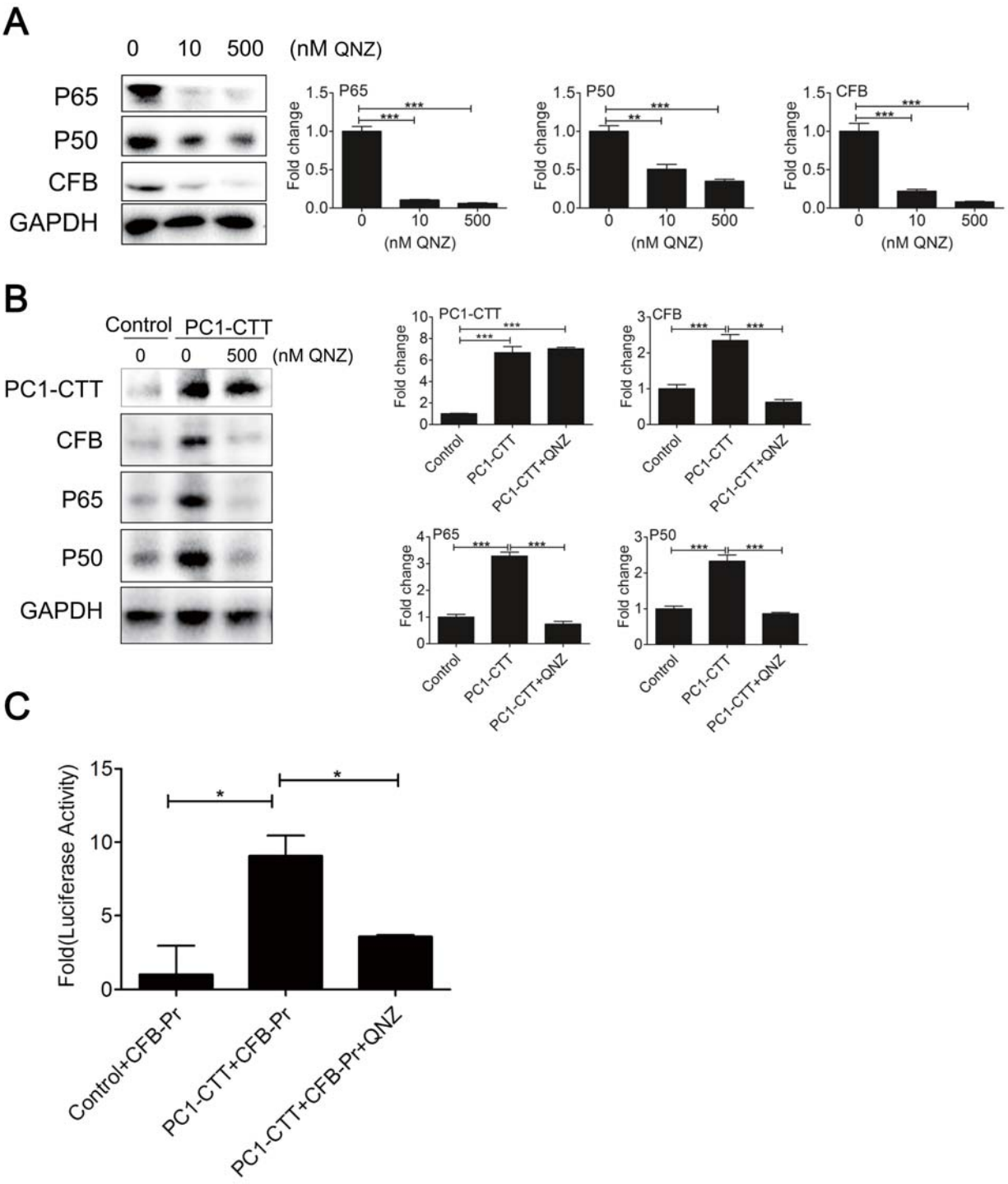

A
Arg1
Mrc1
M2
Relative mRNA level
***
NS
Control
CFB
IL-4/IL-3
INF-γ
B
iNOS
TNF-α
M1
C
Arg-1
PC1-CTT
*
D

Figure 9


A
0 10 500 (nM QNZ)
P65
P50
CFB
GAPDH
P65
P50
CFB
Fold change
(nM QNZ)
B
Control PC1-CTT
0 0 500 (nM QNZ)
PC1-CTT
CFB
P65
P50
GAPDH
PC1-CTT
CFB
P65
P50
Control
PC1-CTT
PC1-CTT+QNZ
C
Fold(Luciferase Activity)
Control+CFB-Pr
PC1-CTT+CFB-Pr
PC1-CTT+CFB-Pr+QNZ